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Identification and Characterization of a New Phospholipase C-like Protein, PLC-L_2

Makiko Otsuki,* Kiyoko Fukami,* Takashi Kohno,† Jun Yokota,† and Tadaomi Takenawa*,[1]
**Department of Biochemistry, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; and †National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

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We have isolated a cDNA encoding a novel protein, PLC-L_2, with homology to the phospholipase C-like protein PLC-L and δ-type phospholipase C. PLC-L_2 contains a relatively well-conserved PH domain, PLC catalytic region, and X and Y domains. However, it did not have PLC activity. This inactivation was thought to be caused by the replacement of two amino acids that are essential for PLC activity, His356 and Tyr552, with Thr and Phe in the X and Y domain. PLC-L_2 has a wide distribution with strong expression in skeletal muscle and mapped to chromosome 3p24–25. The PH domain of PLC-L_2 bound strongly to PI(4,5)P_2 and Ins(1,4,5)P_3, and moderately to PI(4)P and PI(3,4,5)P_3. PLC-L_2 predominantly localized to perinuclear areas in both myoblast and myotube C2C12 cells. Ectopically expressed GFP–PLC–L_2 also mainly localized in perinuclear areas, including endoplasmic reticulum in COS 7 cells. Furthermore, the expression of GFP-PH showed the same intracellular distribution as the full-length PLC-L_2. All these results suggest that PLC-L_2 plays an important role in the regulation of Ins(1,4,5)P_3 around the endoplasmic reticulum on which the Ins(1,4,5)P_3 receptor exists. © 1999 Academic Press

Phospholipase C (PLC) plays an important role in inositolphospholipid signaling by hydrolyzing phosphatidylinositol 4,5-bisphosphate(PI(4,5)P_2), resulting in the formation of two second messengers, inositol 1,4,5-trisphosphate(Ins(1,4,5)P_3) and diacylglycerol. To date ten subtypes of PLC have been found in mammalian species (1–4). On the basis of their structure, they have been divided into three classes, β(β1–4), γ(γ1 and 2) and δ(δ1–4) types (5–8). All these PLCs have X and Y domains that are catalytic sites for PLC activity (9). However, a new type of PLC, which does not belong to either of these three classes has been found. This PLC, referred to PLC210, was first found in nematoda (10). PLC210, whose molecular weight (210 kDa) is higher than that of any other type of PLC, has an Ras binding site and Cdc25-homologous region in addition to X and Y domains. This protein was shown to have PLC activity. Further, another PLC-like protein has been isolated, PLC-L (11). PLC-L is very homologous to PLC δ1 in structure. Its molecular weight (130 kDa) is greater than that of the δ type PLCs but less than that of the β type and γ type PLCs. PLC-L has a PH, X and Y domain and C2 region as δ type PLC (10–13). The PH domain has binding ability for PI(4,5)P_2 and Ins(1,4,5)P_3 like the δ type PLC, which is important for anchoring to plasma membranes in response to extracellular stimuli and detaching from plasma membranes in response to an increase in Ins(1,4,5)P_3 (14–18). However, PLC-L was found not to have PLC activity (12). Thus, it is still unclear what physiological roles are played by this PLC-like protein.

Here we found another type of PLC-L, PLC-L_2, which has a PH, X and Y domain but no PLC activity. The PH domain of PLC-L_2 binds PI(4,5)P_2 most strongly among various inositolphospholipids, and binds Ins(1,4,5)P_3 most markedly among various inositolphosphates.

Abbreviations used: PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphates; PLC, phospholipase C; PH, pleckstrin homology; GFP, green fluorescent protein.

[1] To whom correspondence should be addressed. Fax: 81-3-5449-5417. E-mail: takenawa@ims.u-tokyo.ac.jp.

MATERIALS AND METHODS

Phosphatidylcholine, phosphatidylserine, PI, PI(3)P and PI(3,4)P_2 were purchased from Doosan Sedary Research Labs. [^{3}H]PI(4,5)P_2 and [^{3}H]Ins(1,4,5)P_3 were from Dupont-New England Nuclear. PI(4,5)P_2 and PI(4)P were purified from bovine spinal cords (19).

cDNA cloning and sequencing. The open reading frame was cut out from PLC-L cDNA, labeled with [α-^{32}P]dCTP, and used as a probe for screening a human brain cDNA library constructed in λZAPII (Stratagene). The positive clones were subcloned into the *EcoR* I site of pBluescript KS(−) and sequenced. However, the clones did not cover the open reading frame of PLC-L_2 cDNA. Further, a mouse myoblast λgt10 cDNA library was screened using the human PLC-L_2

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A

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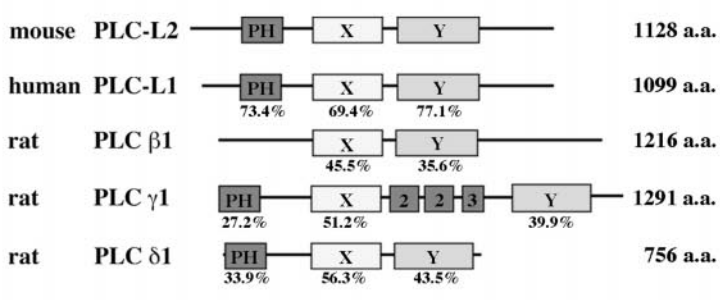

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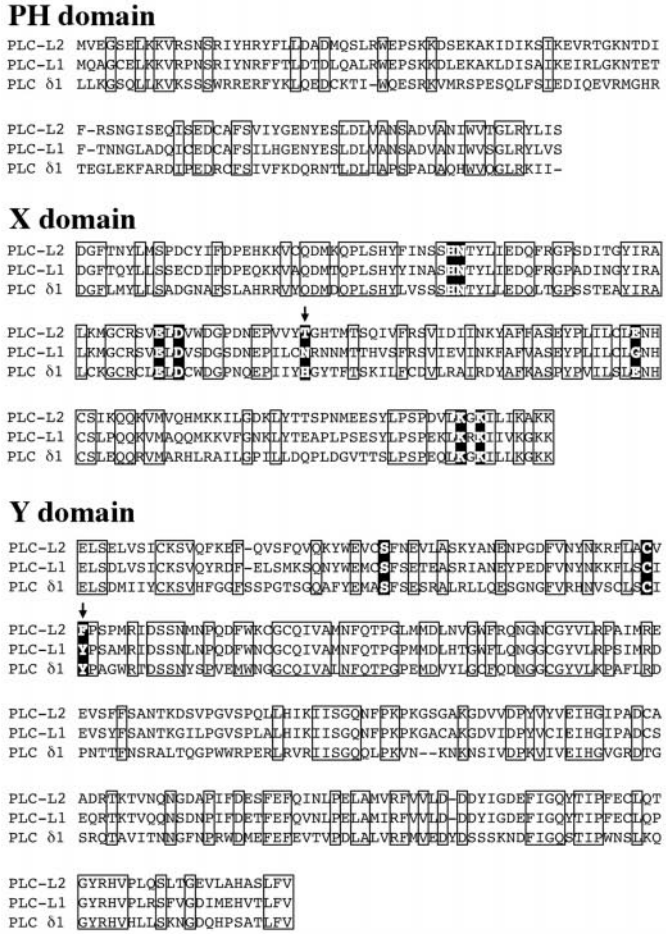

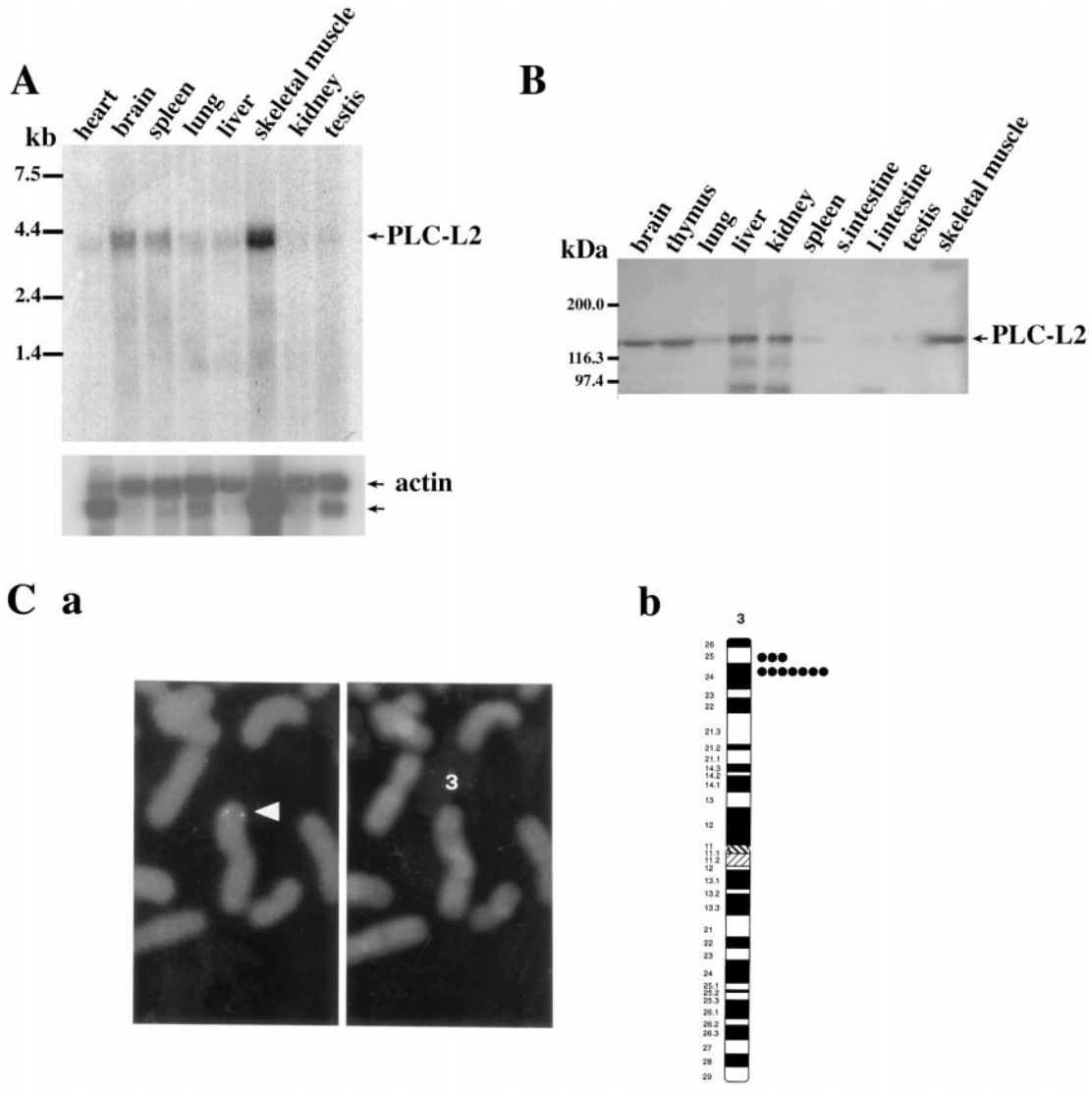

FIG. 2. Tissues distribution of PLC-L_2. (A) Northern blot analysis of 2 μg of mRNA from mouse tissues detected by PLC-L_2 cDNA and β-actin cDNA. (B) Western blot analysis of 20 μg of mouse tissues lysates detected by the affinity purified polyclonal PLC-L_2 antibody. (C) Human chromosome map for PLC-L_2 by FISH; (a) example of FISH mapping of probe PLC-L_2; (left) the FISH signals on the chromosome; (right) the same mitotic figure stained with DAPI to identify chromosome 3; (b) diagram of FISH mapping results for probe PLC-L_2. Each dot represents the double FISH signals detected on human chromosome 3.

cDNA as a probe. The positive clones were cut out from the vector with *Not* I and sequenced.

Ectopic expression of PLC-L_2 and its PH domain. The full length cDNA of mouse PLC-L_2 was ligated into the *BamH* I site of pEGFP mammalian expression vector (Clonetech). For the expression of the PH domain, polypeptides containing the PH domain (amino acids 128–358) were constructed by polymerase chain reaction. The polymerase chain fragment was ligated into the *BamH* I site of pEGFP vector.

Northern blotting. Multiple northern blots of mouse (Clontech) were hybridized with ^{32}P-labeled probes according to the manufacturer's protocol. The C-terminal region (nucleotide 3280–3990) of mouse PLC-L_2 cDNA and control actin cDNA (Clontech) was used as probe.

FIG. 1. The predicted amino acid sequence of PLC-L_2 and a comparison with PLCs. (A) The predicted amino acid sequence of PLC-L_2. (B) Linear display of PLC-L_2, PLC-L_1, and three types of PLCs. PLC β, PLC γ, and PLC δ are represented by β1, γ1, and δ1 isozymes. Numbers under domains indicate homology to PLC-L_2. PH, X, Y, 2, and 3 represent PH domain, X domain, Y domain, SH2 domain, and SH3 domain, respectively. (C) Alignment of amino acids sequences corresponding to PH, X and Y domains. The predicted amino acid sequence of the PLC-L_2 protein is compared with the human PLC-L_1 protein and the rat PLC δ1 protein. Identical amino acids are boxed. Amino acids shown as white letters are essential for PLC activity.

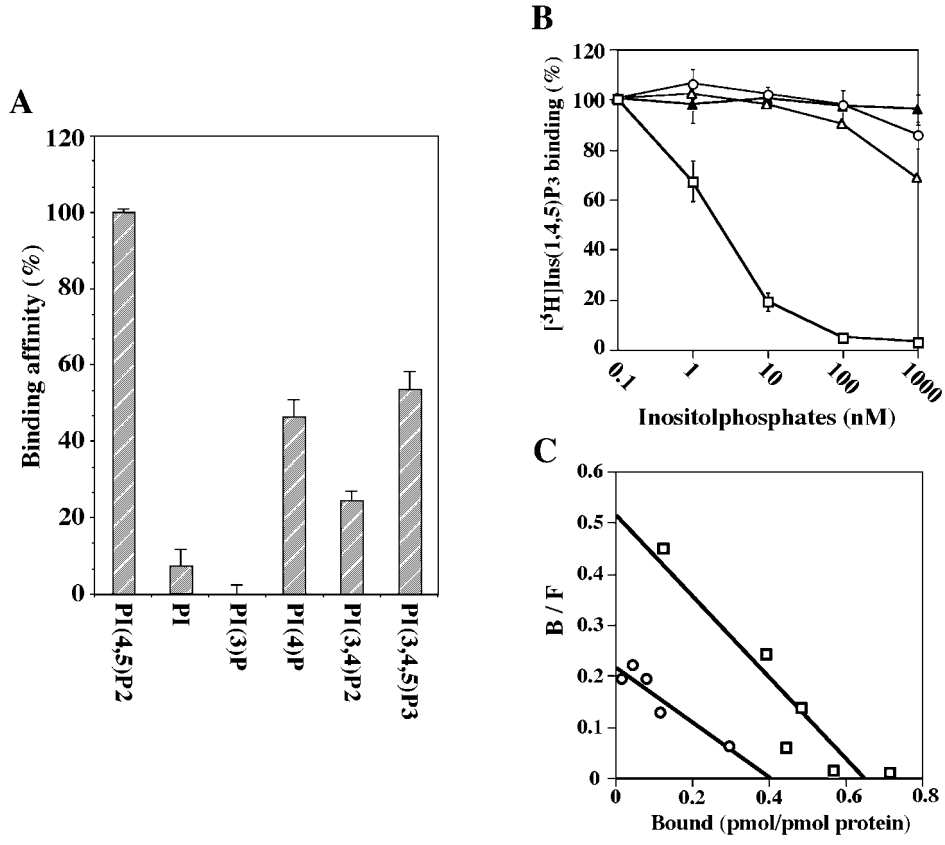

FIG. 3. The binding activity of the isolated PH domain of PLC-L_2. (A) Binding activity of PLC-L_2 PH domain for various inositolphospholipids. The activity of PH domain for $PI(4,5)P_2$ equals 100%. The results are averages of two independent experiments. (B) Binding specificity of PLC-L_2 PH domain for various inositolphosphates. [^{3}H]$Ins(1,4,5)P_3$ binding to PH domain was assayed in the presence of various concentrations of inositolphosphates. □, $Ins(1,4,5)P_3$; △, Ins4P; ○, $Ins(3,4)P_2$; ▲, $Ins(1,3,4,5)P_4$. Each point is the average of two independent experiments. (C) Scatchard analysis of $PI(4,5)P_2$ and $Ins(1,4,5)P_3$ binding to PH domain. Circles and squares represent the binding for $PI(4,5)P_2$ and $Ins(1,4,5)P_3$, respectively. The results are averages of two independent experiments.

GST–fusion proteins. Glutathione S-transferase fusion proteins of the PH domain (amino acids 128–358) was constructed in pGEX plasmids (Pharmacia). The protein was expressed in *E. coli* and purified by glutathione column.

Mapping on chromosomes. Chromosomal mapping was performed as described (20, 21). Human PLC-L_2 cDNA fragment (2.45-kb) was used as a probe.

Binding assay for inositolphospholipids. The purified GST-fusion proteins containing the PH domain were dialyzed in a buffer (20 mM HEPES, pH 7.4, 120 mM NaCl, 2 mM $MgCl_2$). The protein was mixed with micelles containing 40 μM [^{14}C]phosphatidylcholine and 10 μM various inositolphospholipids (PI, PI(3)P, PI(4)P, $PI(4,5)P_2$, $PI(3,4)P_2$ and $PI(3,4,5)P_3$) in 50 μl of buffer for 30 min at room temperature. Then 30 μl of glutathione Sepharose beads was added and the tubes were incubated for 15 min with occasional mixing. After 150 μl of the buffer had been added, the tubes were centrifuged and the supernatant was discarded. The beads were then washed with another 150 μl of the buffer, and the radioactivity bound to the beads was measured.

Binding assay for inositolphosphates. The purified GST-fusion proteins were incubated with [^{3}H]$Ins(1,4,5)P_3$ in 50 μl of buffer for 30 min at room temperature. Then 10 μl of bovine γ-globulin (30 mg/ml) and 1 ml of 20% polyethyleneglycol 6000 were added, and the tubes were incubated for 30 min on ice. After a centrifuge at 100,000 × g for 10 min, the precipitates were dissolved in 100 μl of 0.1 N NaOH, and then the radioactivity was measured.

Intracellular localization of PLC-L_2. C2C12 cells grown on glass coverslips were fixed in 3.7% formaldehide, washed with phosphate-buffered saline (PBS) and incubated in 0.2% Triton X-100. Then the coverslips were incubated with the affinity purified polyclonal PLC-L_2 antibody for 1 h. FITC-conjugated rabbit IgG antibody was used as secondary antibody.

RESULTS AND DISCUSSION

Cloning of mouse PLC-L_2 cDNA. Using human PLC-L cDNA (11) as a probe, cross hybridization screening of a human brain cDNA library was carried out and five positive clones were obtained. Sequencing revealed 4 clones to be PLC-L itself and the other to encode a 2.45 k-bp cDNA, which was found to be the same as KIAA1092 (22). Since this last clone was thought to be a homolog of PLC-L, we named it PLC-L_2 and renamed the original PLC-L, PLC-L_1.

Using this human PLC-L_2 cDNA fragment, we performed fluorescent in situ hybridization to determine the chromosomal localization of PLC-L_2. PLC-L_2 was mapped to chromosome 3p24–25 (Fig. 2C). Since human PLC-L_1 was reported to localize at chromosome

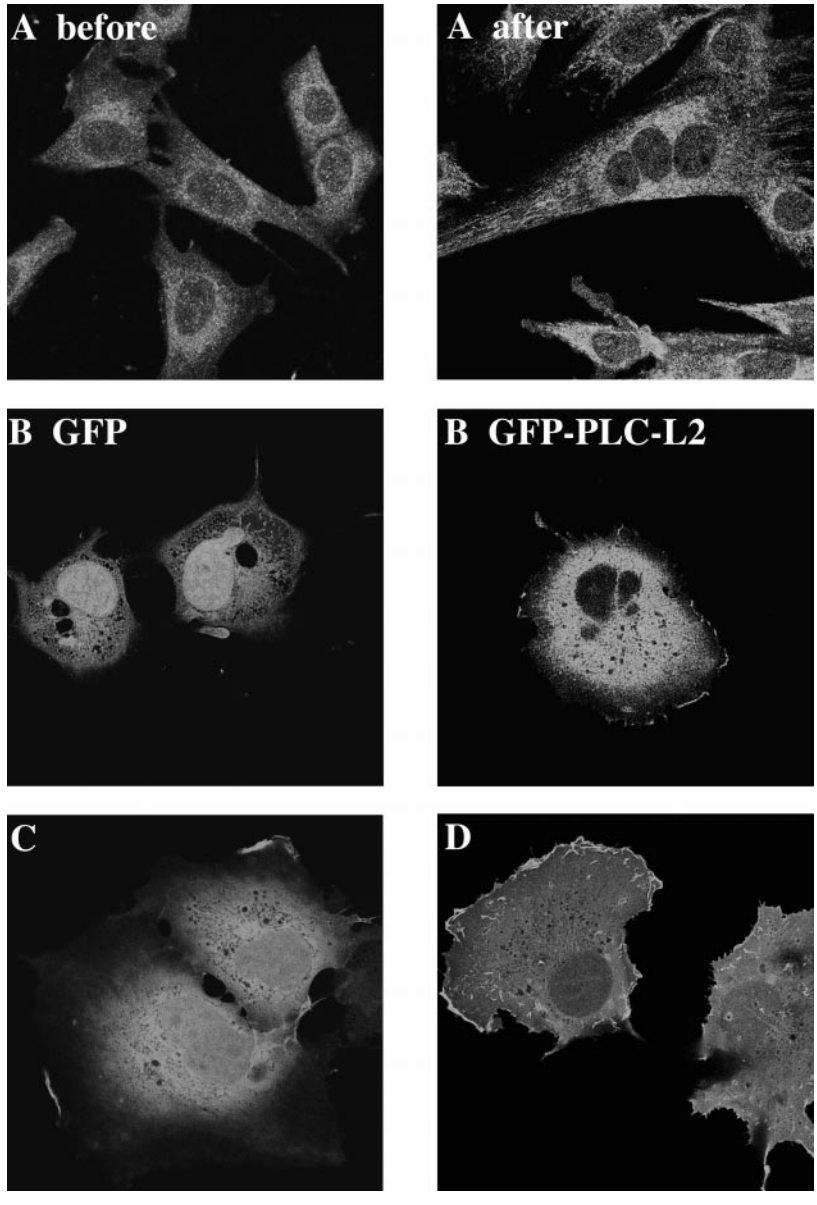

FIG. 4. (A) Intracellular localization of endogenous PLC-L_2. Immunoreactive PLC-L_2 before and after differentiation in C2C12 cells was detected by the affinity purified polyclonal PLC-L_2 antibody. (B) Intracellular localization of exogenous PLC-L_2. GFP and GFP-PLC-L_2 plasmids were transiently expressed in COS 7 cells. (C, D) Intracellular localization of PH domains. GFP-PH domain plasmids of PLC-L_2 (C) and PLC δ1 (D) were transiently expressed in COS 7 cells.

2q33 (11), it is clear that PLC-L_2 is not a splicing isoform of PLC-L_1, but derived from a different gene.

However, this PLC-L_2 cDNA was not of full length. Then we further screened a mouse myoblast cDNA library with this human PLC-L_2 cDNA and obtained a 4185 base pair-long mouse PLC-L_2 cDNA. This gene had a stop codon 190 bp upstream from the putative first methionine, and the sequence surrounding this methionine followed the Kozak rule (23). Thus, we concluded that this open reading frame encoded a putative mouse PLC-L_2 protein, which consists of 3384 base pairs encoding 1128 amino acids (Fig. 1A) (DDJB/EMBL/GenBank accession number, AB033615).

The mouse PLC-L_2, just like human PLC-L_1, had X and Y domains which are essential for PLC activity and a PH domain. Comparison of the entire amino acid sequence showed that mouse PLC-L_2 has 63.9% identity to human PLC-L_1. The X, Y and PH regions in particular were highly conserved with 69.4%, 77.1% and 73.4% amino acid identity, respectively (Fig. 1B). But this PLC-L_2 had an extra 29 amino acids at the N-terminus compared to PLC-L_1. Furthermore, this

protein was homologous to the PLCs (Fig. 1B): it was most homologous to δ type PLC in amino acid sequence and structure. We next examined whether 11 amino acids (shown as white capital letters in Fig. 1C) in the X and Y domain, which are thought to be essential for PLC activity from the structural analysis of PLC δ1, are conserved in PLC-L_2 (9, 24). Among these amino acids, His356 and Tyr551 (indicated by arrows in Fig. 1C) were replaced by Thr and Phe, respectively. These results suggest the possibility that PLC-L_2 does not have PLC activity. Thus, we checked whether PLC-L_2 has PLC activity using immunoprecipitates of PLC-L_2 from lysates of PLC-L_2-expressed COS 7 cells or proteins expressed by the baculovirus system. But we did not detect PLC activity against PI(4,5)P_2, PI(4)P or PI (data not shown).

Tissue distribution of PLC-L_2. To analyze the tissue distribution of PLC-L_2, we performed Northern blot analysis. PLC-L_2 was highly expressed in skeletal muscle and moderately expressed in brain and spleen (Fig. 2A). Next, we carried out Western blot analysis using various mouse tissues. The PLC-L_2 protein band was detected at 130 kDa, which is similar to the molecular weight (124 kDa) estimated from its open reading frame. PLC-L_2 was predominantly distributed in skeletal muscle but also present in brain, thymus, kidney and liver (Fig. 2B).

Inositolphospholipids and inositolphosphates binding to the PH domain. PLC-L_2 has a PH domain at the N-terminus, in which 73.4% and 33.9% of amino acids are identical to those of PLC-L_1 and PLC δ1, respectively (Fig. 1B) (12, 25, 26). Since the PH domain is thought to be a binding site for inositolphospholipids and inositolphosphates, we studied the binding character of the PLC-L_2 PH domain for these molecules. The PH domain bound most strongly to PI(4,5)P_2 and moderately to PI(3,4,5)P_3 and PI(4)P among various inositolphospholipids (Fig. 3A). Next, we studied the binding ability of various inositolphosphates by the competitive inhibition of [^{3}H]Ins(1,4,5)P_3 binding. As shown in Fig. 3B, [^{3}H]Ins(1,4,5)P_3 binding was only inhibited by Ins(1,4,5)P_3, and not by Ins(4)P, Ins(3,4)P_2, Ins(4,5)P_2 or Ins(1,3,4,5)P_4.

Since the PLC-L_2 PH domain binds strongly to PI(4,5)P_2 and Ins(1,4,5)P_3, the dissociation constant, Kd value, was measured against these molecules. According to the scatchard analysis, we determined that the Kd value was 3.7 μM for PI(4,5)P_2, and 2.5 μM for Ins(1,4,5)P_3 (Fig. 3C).

Intracellular localization of PLC-L_2. The intracellular localization of endogenous PLC-L_2 was examined using a polyclonal antibody against PLC-L_2. We immunostained C2C12 cells, a mouse myoblast cell line because PLC-L_2 is highly expressed in skeletal muscle. A dot-like pattern was obtained throughout the cytoplasm both in undifferentiated myoblast and differentiated myotube cells. When GFP-PLC-L_2 was expressed in COS 7 cells, it was present throughout the cytoplasm but also concentrated in perinuclear areas (Fig. 4A, B). Further the localization of the PH domain was compared to that of PLC δ1 using GFP-tagged PH domains (27, 28). The PH domain of PLC δ1 predominantly localized to plasma membranes supporting the notion that this PH domain is anchored to PI(4,5)P_2 at plasma membranes. However, the PH domain of PLC-L_2 localized markedly to perinuclear areas though it was also present at plasma membranes (Fig. 4C, D). These results suggest that PLC-L_2 localizes around the endoplasmic reticulum, on which the Ins(1,4,5)P_3 receptor exists, and interferes with Ins(1,4,5)P_3 signaling (29, 30).

PLC-L_2 does not have PLC activity though it has a structure similar to PLC δ1. Therefore, it may regulate inositolphospholipid signalling since it still has the capability to bind inositolphospholipids and inositolphosphates like δ type PLCs (25). In this case, this protein interferes with Ins(1,4,5)P_3 metabolism more than dose PI(4,5)P_2 because it localizes around the endoplasmic reticulum and PI(4,5)P_2 binding seems to be weaker than that of PLC δ1.

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